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RESEARCH ARTICLE

Chemical analysis and protein enzymatic hydrolysis of poly-floral bee pollen

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ABSTRACT

This study assessed chemical components and enzymatic hydrolysis of bee pollen (BP) as an approach to modify its structure and generate bioactive peptides which can be used as functional ingredients. The food grade proteases Alcalase 2.4L and Protamex were used at optimized parameters. The degree of hydrolysis (DH), protein content, and molecular mass (MM) distribution of the soluble peptides in the hydrolysates were determined as indicators of enzymatic hydrolysis. The highest DH was reached by Alcalase 2.4L (10.39 %), whereas Protamex showed the lowest (9.31 %). The total protein content recovered from the hydrolysates was 16.01 % w/w for Alcalase 2.4 L and 15.51 % w/w for Protamex. The Electrophoretic profile for Alcalase 2.4L and Protamex showed a range of low molecular (4.5 kDa) and low – medium mass peptides (5.8-51 kDa), respectively. Protein recovery and analysis of the MM distribution of the peptides provide data for further research, taking into account the trend of using BP peptides in food products.

Keywords: Bee pollen; Chemical analysis; Enzymes; Hydrolysis; Proteins

INTRODUCTION

The global demand for foods with high-quality proteins derived from plants has considerably increased in recent years. The FAO expects a 40 % increase in protein demand by 2030 for two reasons: 1. in response to global population growth, which is estimated to reach 8.4 billion; 2. the nutritional transition, which means switching from a diet containing a small amount of protein to a diet high in proteins (Gaudichon et al., 2021). This challenge can be overcome by diversifying and searching for alternative protein sources for human consumption.

Bee products include honey, pollen, bee bread, propolis, royal jelly, and bee venom. BP, also known as apicultural, bee-collected or corbicula pollen, is a product of the hive consisting of pollen from plant anthers to which flower nectar is added, regurgitated honey and saliva secreted by the pharyngeal and thoracic glands of bees, which is rich in enzymes (amylases, catalases) (Thakur et al., 2020). The amount of pollen collected by one colony of bees is 50-250 g/day and from 15 to 40 kg of pollen/year. As weight measure, about 15,000 pollen grains weigh ~ 1 g (Komosinska-Vassev et al., 2015).

BP has gained increasing interest in recent years, not only for its nutritional value, but also for its physiological properties on the human body. Pollen from different plant species contains more than 250 valuable biologically active substances. The BP value is given by the high content of carbohydrates (mainly fructose, glucose, sucrose), proteins, amino acids (proline can exceed half of the total free amino acids), fatty acids (linoleic acid, γ-linolenic acid, and arachidonic acid), vitamins (mainly B-complex), pigments (chlorophyll, carotenoids), minerals (Zn, Cu, Fe, K, P, Ca, Mg), polyphenols (flavonoids, phenolic acids), and traces of micronutrients. The chemical composition of pollen depends strongly not only on the plant source but also on geographical origin, climatic conditions, soil type, bees' race and activities (Kieliszek et al., 2018; Taha et al., 2019). Due to its composition, BP is considered to be the "only perfectly complete food" (Conte et al., 2018).

Currently, due to food security issues of animal-derived proteins, the interest in pollen as an source of proteins has

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increased (Du et al., 2017). Proteins, amounting to 22.7 % on average, contain eighteen amino acids, including all the essential amino acids needed for the human organism (Conte et al., 2018). The content of amino acids in the pollen collected by bees in the spring is significantly different compared to the pollen load collected in the summer (Kieliszek et al., 2018). Reference protein (egg protein was used as a standard for evaluating proteins from BP) value for pollen is 33.9 % and estimates amino acid requirements expressed in accordance with the safe level of protein intake. From a comparison of the amino acid pattern (the percentage of total essential amino acids to total amino acids in BP) with the reference pattern Millward et al., 2012), it turned out that the BP may be a "health-promoting" source of high-quality protein (Thakur et al., 2018). From this perspective, pollen can be considered as a potential vegetal source of protein if taking into account the consumer requirement for a healthy diet and the many possibilities of application in the food industry (Maqsoudloua et al., 2019). A group of researchers (Conte et al. 2018; Zuluaga et al., 2016) starting from the promising results regarding the incorporation of BP in various food products (biscuits, bread) have proposed the creation of a platform for the future processing of BP.

The pollen pellets collected by bees undergo changes in chemical composition which leads to the improvement of its therapeutic qualities. Experimental pharmacological and clinical studies demonstrated that BP exhibits antioxidant, antimicrobial, antihypertensive, anti-inflammatory, anticarcinogenic, antidiabetic and antiallergic properties. The antioxidant activity is the most important and is attributed to polyphenolic compounds which have the intrinsic ability to reduce reactive oxygen species (ROS). ROS are composed of free radicals that are extremely reactive with proteins, lipids, and DNA, regarding their unpaired electrons, and which are oxidatively degraded (Demir et al., 2019). The pathological processes reported to be associated with ROS are apoptosis, aging, diabetes, cell injury, cancer and inflammation (Aslan et al., 2017) and the incriminating factors are obesity, smoking, alcohol consumption, stress and air pollution (Demir et al., 2019). That is why its consumption as a dietary supplement and applications in the medical field are constantly growing (Li et al., 2018).

For human consumption, the accessibility of valuable pollen components is limited by the presence of sporopollenin exine, which covers pollen grains on the outside. Enzymatic hydrolysis is an effective method that can be applied to BP in order to release high value-peptides by structurally modifying the exine.

There are few studies regarding enzymatic hydrolysis of BP and peptide generation. Most of the reported studies have focused on mono-floral BP from different geographical regions following the physical – chemical, nutritional (Thakur et al., 2018; Liolios et al., 2019), antibacterial and antioxidant (Marghitas et al., 2016; Spulber et al., 2017; Kostić et al., 2019) and functional (Kostić et al., 2015) properties. However, to the best of our knowledge, few studies on autochthonous poly-floral BP have been conducted. They are focused on determining the nutritional and biological value of BP (Margaoan et al., 2012), and assess the antibacterial activity of pollen ethanolic extracts over bacteria in milk (Marghitas et al., 2016; Spulber et al., 2017). A study on the enzymatic hydrolysis of BP from floral combination and analysis of the MM distribution of the peptides has not been found in the literature.

In this respect, the aim of this study was a) to characterize the BP from local poly-floral sources, from a chemical point of view; b) to obtain and compare hydrolysates from BP using two different commercial enzymes, selected based on their specific activity, accessibility and relative low-cost price; c) to evaluate the enzymatic hydrolysates by determining the degree of hydrolysis (DH) and electrophoretic pattern (peptides molecular profile).

MATERIALS AND METHODS

Chemicals and reagents

Raw BP used in all experiments was obtained from ApiLand SRL, a local brand. The sample of raw BP is a mixture of pellets of different shapes and colors, which come from several floral species: wild rose, sweet chestnut, willow, black grass, common hawthorn, mountain flowers, plum, dandelion, apple, cherry, blue eryngo. The pollen

Table 1: Characteristics of the commercially available enzymes* used in this study

Comercial brand	Enzyme type	Biological source	Number EC	pH range	Optimal pH	Temperature range	Activity units
Alcalase 2.4L	Serin endoprotease	*Bacillus licheniformis*	3.4.21.62	6.5-9	8.5	30°C - 65°C	2.4 AU/g
Protamex	Serin endoprotease/ neutral metallo-endopeptidase	*Bacillus amyloliquefaciens*	3.4.21.14	5-11	7	30°C - 65°C	1.5 AU/g

*A.S. Novozymes, Product Sheet ProtamexTM, 2002 [12 July 2010]. Available at: www.novozymes.com; Novozyme – Bio-catalysis brochure - Proteases for bio-catalysis

samples were grounded and manually kept in a freezer at a temperature of -18 ^{0}C. Food grade enzymes Alcalase 2.4 L and Protamex were obtained from Novozymes (Bagsvaerd, Denmark) (Table 1).

The reagents used in this study were analytical grade available and commercially. All solutions were prepared with ultrapure water (Milli-Q® INTEGRAL system). Reagents used in electrophoretic separation were supplied by Invitrogen by Thermo Fisher Scientific, USA.

Proximate chemical analysis of BP

The proximate BP includes moisture (AOAC 925.10/2005), total nitrogen (Kjeldahl method), total protein (result obtained by calculation), lipids (AOAC 920.39/2005), ash (AOAC 900.02/2007), mineral content (AOAC 920.181/2005) (Inductively coupled plasma optical emission spectrometry- ICP-OES), carotene (HPLC), polyphenolic compounds (liquid chromatography coupled with mass spectrometer analyzer and time-of-flight detector - TOF LC/MS).

Pre-treatment

The finely ground raw BP grains were diluted 1:4 with ultrapure water and vortexed to obtain a homogeneous slurry. The samples were placed in a porcelain capsule containing ice and treated by ultrasound (Sonoplus Ultrasonificator model CV 334), at 750 W, 70 % amplitude, 20 kHz frequency, 30 min (the pulse duration of on-time 3 s and off-time 2 s). After that, the samples were homogenized (Ultraturax IKA T18 basic homogenizer) at 25000 rpm for 1 min.

Enzymatic hydrolysis of BP protein

Enzymatic hydrolysis of BP was performed with two proteolytic enzymes, Alcalase 2.4L and Protamex, according to the method described by (Maqsoudloua et al., 2019) with modifications. Briefly, after pre-treatment, the BP solutions were adjusted and initially to the optimal value of pH, appropriate for each enzyme, by adding NaOH 1N. The samples were incubated one by one (Nahita Refrigerated Incubator Model no. 639/70) at pH 8.5, 55 ^{0}C for Alcalase 2.4L and pH 7, 55 ^{0}C for Protamex. So, when the set parameters were reached, the hydrolysis process begins by adding the enzymes, with continuous gentle stirring, in concentrations of 0,55 U/g and 0.307 U/g of protein content in the substrate, respectively. The enzymes were added in such amounts to have equal proteolytic activity. Aliquots (10 mL) were taken from the mixtures after 30, 60, 150 and 240 min to determine the DH. Throughout the hydrolysis the pH was kept constant. The reaction was stopped by heating the samples at 100 ^{0}C for 10 minutes for enzymes inactivation. Untreated samples (fresh pollen) of BP were used as a control similarly. After cooling to room temperature and neutralization, the obtained hydrolysates were centrifuged at 9000 rpm, for 30 min, at 4 ^{0}C to separate the unhydrolyzed residue from the soluble hydrolyzed material (protein phase). The supernatants were collected, lyophilized and kept at -20 ^{0}C. All experiments were performed in duplicate.

Protein content

The total protein content was determined using the Kjeldhal method. The solubilized nitrogen in the supernatants was determined and the total nitrogen in the soluble fractions was calculated following the equation (1):

Nitrogen % = total nitrogen in supernatant/total nitrogen in substrate sample (1)

Total protein in the supernatant was computed taking 5.60 as nitrogen conversion factor. The plant material contains high concentrations of non-protein nitrogenous substances (chlorophyll, nucleic acids, free amino acids, inorganic nitrogen in the form of ammonia, nitrates, nitrites) which contribute to an overestimation of the protein content if the conversion factor 6.25 is used (Ezeagu et al., 2002). All assays were done in triplicate.

The degree of hydrolysis

The DH was determined using soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), method described by Kaewka et al. (2009). An aliquot of 10 mL of the aqueous suspension of the hydrolyzed protein was mixed with 10 mL of 10 % TCA, allowed to stand for 30 min at room temperature, and centrifuged at 4 ^{0}C for 10 minutes at 9000 rpm. The soluble nitrogen in the supernatant was determined by the Kjeldahl method, and the degree of hydrolysis was calculated using the formula in equation (2):

DH (solubility index in TCA) (%) = (total nitrogen soluble in TCA and present in supernatant/total nitrogen in the substrate sample) (x) 100 (2).

Although the SN-TCA method does not determine the DH directly, it has been used in some studies to estimate the DH of pumpkin protein isolate, fish offal, beans, casein and whey protein concentrate (Rutherfurd, 2010). After comparing different DH methodologies, no consensus was reached regarding the best method for determining the DH of protein hydrolysates. Based on literature data, it was found that some methods (pH-stat and formalin titration) do not correlate with SN-TCA, but others (TNBS and OPA) are well correlated. Enzyme, pH, extent of hydrolysis, and protein source and amino acids composition influence the appropriate method. So, the standardization of a protocol

for the correct determination of DH is required.

Fractionation of BP protein hydrolysates by ultrafiltration

The protein hydrolysates were fractionated in an Amicon Stirred Cell (Merck Millipore) using a membrane disc with 100 kDa molecular weight cut-off (Biomax®). The retentate fractions were lyophilized and the permeate was kept at -20 ^{0}C until use.

Determination of the MM distribution

The electrophoretic analysis was performed by SDS-PAGE according to the Laemmli method (Laemmli et al., 1970) with slight modifications, using precast gradient gel (BoltTM 4-12% Bis-Tris Plus, Invitrogen by Thermo Fisher Scientific, USA) and running buffer BoltTM MES SDS Running Buffer (20x), in reducing conditions. Samples of protein hydrolysates and control (fresh pollen) were diluted in Sample Buffer, Laemmli (2x) at a ratio of 1:1 (v/v) to a concentration of 0.5 mg protein/mL and heated at 95 ^{0}C for 10 min to cleave non-covalent bonds. Following centrifugation at 14000 rpm for 5 min, aliquots of 20 µl of each sample solution were loaded in the wells. SeeBlue® Plus2 Pre-Stained Protein Standard (Invitrogen by Thermo Fisher Scientific, USA) ranging in MM from ~ 3 – 198 kDa, was also loaded into the gel to estimate the MM of each protein hydrolysate. Electrophoresis was carried out for approximately 60 min at a constant current of 100 V and at room temperature, in a vertical electrophoresis system (Mini Gel Tank, Invitrogen). For protein subunits (polypeptides, peptides) visualization the gel was stained using 0,1% w/v Coomassie Brilliant Blue R-250, which binds to the proteins. Afterwards, the gel was de-stained by diffusion in a solution of 30 % (v/v) methanol and 10 % (v/v) acetic acid, for 24 h, on shaker, until clear gels were obtained. The gel images were scanned and analyzed. Three assays were performed.

Statistical analysis

Statistical analysis of data was performed with Minitab Version 18 (Minitab, LLC, United States). All results were expressed as mean values ± standard deviation of at least three measurements and (n = 3), Tukey pairwise comparision by mean of the data was conducted using a one-way analysis of variance (ANOVA) for a statistically significant level of $p<0.05$.

RESULTS AND DISCUSSION

Chemical analysis of BP

The sample of raw BP studied was a mixture of glomeruli of different shapes and colors, which come from several floral species. The mean percentage (± SD) of chemical compounds are presented in Table 2.

Table 2: Chemical composition of fresh BP

Parameter	Value	Measure units
Moisture at 105 ^{0}C	28.4 ± 0.5	% (w/w)
Total nitrogen (TKN)	2.79	% (w/w)
Total protein	15.62 ± 1.7	% (w/w)
Ash at 550 ^{0}C	2.26 ± 0.8	% (w/w)
P (λ = 213,617 nm)	0.449	% (w/w)
K (λ = 766,490 nm)	0.532	% (w/w)
Ca (λ = 317,933 nm)	0.155	% (w/w)
Mg (λ = 280,271 nm)	0.081	% (w/w)
Fe (λ = 238,204 nm)	65.8	mg/kg
Na (λ = 589,592 nm)	46.1	mg/kg
Mn (λ = 257,610 nm)	79.9	mg/kg
Zn (λ = 213,857 nm)	39.3	mg/kg
Cu (λ = 327,393 nm)	6.70	mg/kg
Mo (λ = 202,031 nm)	< 2.2	mg/kg
β-Carotene	91.1	mg/kg
Total phenols	28.03	mg GAE/g

All percentages were in w/w dry basis

The water content in the fresh pollen loads falls within the range of 21% to 30% (Kieliszek et al., 2018). The protein content of fresh BP samples was lower than that determined on 22 samples, most of which were found to be hetero- floral pollen from Portugal (21.8 % dry base) (Feás et al., 2012), but falls within the of range 16.27 % - 26.50 % analytically determined for bee pollen of multi-floral origin, harvested from different areas of Transylvania (Romania) (Margaoan et al., 2012). The standard norms of BP physical-chemical and microbiological qualities in Brazil and Argentina provide a minimum of 8 % and between 15-28 % for this parameter, respectively. So, the protein content determined in the analyzed sample characterizes its nutritional value. The higher protein content reported for pollen from other countries is probably related to the different geographical and climatic conditions, but the development of the bees hypopharyngeal glands and ovaries are correlated with the protein portion present in pollen, too. The contribution of the protein content from analyzed local BP to the daily nutritional requirement is 8.7 %.

The mineral content was expressed as ash content and depends on the floral source and the growing conditions (soil, geographical area). The ash determined in the samples was 2.26 % w/w and represents an important quality index. If this parameter exceeds the maximum value (over 4%), it is considered that the sample contains mineral impurities and, therefore, it has not been well cleaned.

The most abundant macro-element was potassium (0.532 % w/w), followed by phosphorus (0.449 %) calcium (0.155 %) and magnesium (0.081 %). These

results are slightly higher than those reported by (Feás et al., 2012) for bee pollen in Serbia. Among the microelements, manganese (79.9 mg/kg) predominates, followed by iron (65.8 mg/kg), sodium (46.1 mg/kg) and zinc (39.3 mg/kg). The presence of microelements necessary for the normal functioning of the body (zinc, iron, manganese and copper) recommends pollen in diets with established electrolyte balance (Block et al., 1994). The K/Na ratio was high (115:1) and recommends the use of bee pollen as a food supplement (Campos et al., 2008).

Pollen is a rich source of minerals for which there are daily Nutrient Reference Values (NRVs) in Europe (https://www.efsa.europa.eu/en/topics/topic/dietary-reference-values-and-dietary -guidelines). Mn (99 %), Cu (16 %) and P (16 %) have the biggest contribution to the diet, followed by Fe (11.4 %) and Zn (9.9 %), and Ca (4.8 %) has the smallest contribution. Knowing the mineral composition is very important when using BP as a food supplement. The results obtained claim that pollen is a rich source of mineral elements.

Polyphenols are another category of substances with antioxidant properties. The total amount of polyphenols in ethanolic extracts of 1 g of BP was 28.03 mg GAE/g of dry extract. The polyphenols, their derivatives and metabolites have been identified based on the molecular masses ionized in the electric field, namely: phenolic acids (hydroxycinnamic acid derivatives – caffeic acid and its chlorogenic acid ester, cinnamic acid, ferulic acid; hydroxybenzoic acid derivatives - protocatechuic acid, vanillic acid, gallic acid) and flavonoids (kaempferol, epicatechin, quercetin and its isorhamnetin metabolite, rutin).

The intensity of the ions in the analyzed sample is presented in Table 3.

The results represent the distribution of the compounds identified and nominated in the table, excluding the weight of other existing signals in the sample.

Table 3: The intensity of the ions in the analyzed sample

Compound name	Abundance	Abundance compounds (%)
Cinnamic acid	63685.6	17.61
Protocatechuic acid	28577.5	7.90
Vanillic acid	37004.9	10.23
Gallic acid	28707.2	7.94
Caffeic acid	25082.8	6.94
Ferulic acid	47197.6	13.05
Quercetin	28781	7.96
Chlorogenic acid	47520	13.14
Rutozid (rutin)	55112.1	15.24
Total	361668.7	100

The concentration of β-carotene determined in fresh BP was 91.1 μg/g. The carotenoid pigments are involved in the scavenging of the reactive oxygen species singlet molecular oxygen ($^1O_2$) and peroxyl radicals. Carotenes proved to be efficient quenchers of singlet oxygen due to the number of conjugated double bonds present in the molecule which determines their lowest triplet energy level. β-Carotene has triplet energy levels close to that of $^1O_2$ enabling energy transfer (Stahl et al., 2003).

Protein content

In most reports (Aspmo et al., 2005; Szucs et al., 2021), comparative enzyme studies use the same mass fraction of enzymes in the g to the substrate, regardless of the fact that the enzymes have different specific activities, and optimal working conditions. In our study the specified proteolytic activities are measured at the optimum pH and temperature conditions for the two enzymes. The Proteolytic activity of the enzymes was determined using azocasein as substrate according to (Junior et al., 2020) at the chosen pH and temperature conditions (data not shown).

An important parameter of enzymatic hydrolysis efficiency is protein recovery, which correlates with protein solubility. Protein nitrogen determination in the soluble fractions shows the protein content of the enzymatic hydrolysates. The total protein content in the enzymatic hydrolysate of BP obtained with the enzyme Alcalase 2.4 L reached 16.01% w/w compared to 15.51 % w/w obtained with Protamex and 15.62 % w/w, the value of protein from fresh BP.

The increase in the total amount of protein following enzymatic hydrolysis with Alcalase 2.4 L, compared to fresh BP untreated ultrasonically, could be explained by sample pre-treatment with ultrasonication. The combination of enzymatic hydrolysis with ultrasound facilitates the disintegration of exine and intine and the access of enzymes to intracellular compounds. The hydrogen bonds and hydrophobic interactions are affected and the protein structures are disrupted by ultrasound cavitation effect (Cheng et al., 2017). The application of homogenization after ultrasound led to the massive fragmentation of the cell walls at the level of the germinal openings and implicitly to the release of the components, including a large number of peptides and free amino acids that are inside and which become more accessible in the reaction mixture (Bruno et al., 2019). The unfolding of protein molecules makes enzymes binding sites more exposed and allow the release of compounds at low temperatures at which enzymes are active (Zuluaga-Domínguez et al., 2019). By enzymatic hydrolysis, the pollen proteins acquire commercial and nutritional value.

The results obtained in this study were lower than those reported by (Zuluaga-Domínguez et al., 2019) for the enzymatic hydrolysates of BP collected from apiaries in Colombia, which had a total protein content of 25.32 % w/w and 29.12 % w/w when using the enzymes Alcalase 2.4L and Protamex, respectively. Taking into account the highest result in the content of protein in the hydrolysate, Protamex was more efficient, being in contrast with our results. In a study published by Hu et al. (2007), regarding rapeseed pollen, the protein content mentioned is around 22.4 %. The author demonstrated that the main proteins are glutelin (55.7 %), albumin (39 %), globulins (3.2 %) and prolamin (2.1 %). Glutelin, the predominant protein that are soluble in alkaline media, has been hydrolyzed with Alcalase 2.4L to give biopeptides with antioxidant properties. Other authors (Zheng et al., 2015) used Protamex for the hydrolysis of corn pollen glutelin. It was observed that the MM of glutelin decreased and the insoluble native aggregates became soluble, which supports the effectiveness of Protamex in the hydrolysis of glutelin. Kristinsson et al. (2000) processing fish protein hydrolysate, stated that Alcalase 2.4L was the most effective enzyme to produce a high protein content with a protein recovery of 70.6 %.

The reasons of low protein content in the enzymatic hydrolysate could be: the low protein concentration (15.62% w/w) of fresh BP samples used in this study; insufficient degradation of the outer exine layer of the pollen, which limits the access of enzymes; a high level of solid to liquid ratio (1:4) diluted the sample; enzyme to substrate ratio probably inadequate, either too low which means insufficient catalytic sites to enhance the hydrolysis process, or too large, which can cause the aggregation of enzymes and the increase in substrate diffusion inhibition. On the other hand, after centrifugation, which was done only once, some soluble compounds remained in the residual solids (pellets). Some authors (Hong et al., 2019) stated that the small amount of protein recovered indicates an increase in protein cleavage. The increase in the number of exposed hydrophobic side chains shows a high hydrolytic activity.

It is also worth mentioning that protein hydrolysates (supernatant) were dark in color due to for the co-extraction of pigments and polyphenols that dissolve in the medium with alkaline pH, bind to proteins and are further oxidized into highly colored products. In according with previous studies, proteases significantly influence the release of phenolic compounds (Zuluaga-Domínguez et al., 2019).

Because of this, the quality of the pollen protein and sensory properties of the final products are affected (Zhang et al., 2020).

PROTEIN ANALYSIS

The degree of hydrolysis

DH is the most common proteolysis-indicator and is used to determine the extent and the efficiency of protein peptide bonds breakdown (Venkatachalam et al., 2019). After the ultrasonic pre-treatment and homogenization, the BP was subjected to hydrolysis by two commercial food grade proteases to their optimal parameters, according to previous optimization. The DH values (Table 4), calculated through soluble protein content method, ranged from 5.57 % to 10.39 % for Alcalase 2.4L and 6.71 % to 9.31 % for Protamex.

Results are expressed as the mean ± standard deviation (n=3). Means that do not share a letter are significantly different ($p < 0.05$). The f-ratio value is 10.62. The p-value is 0.022, the result is statistically significant different. The results are significantly different for hydrolysis time 30 vs. 240, which suggests that increasing the hydrolysis time leads to an increase in the degree of hydrolysis, but there is no significant difference between the results obtained for the two enzymes (One-way ANOVA using Tukey pairwise comparison by mean).

The kinetics of hydrolysis had four phases. In the first 60 min the reaction rate was fast, which corresponds to a breakage of a large number of peptide bonds, with accumulation of soluble peptides in the reaction mixture (Nguyen et al., 2011). Thereafter, for both enzymes, a linear decrease in the hydrolysis rate was noticed up to 150 min, which may be due to the much slower hydrolysis of compact or partially digested proteins which competes with the soluble peptides obtained in the initial phase of hydrolysis (Nchienzia et al., 2010), inhibition of the enzyme by the products and formed, which act as substrate competitors (Soussi et al., 2007), enzyme autodigestion (Adler-Nissen et al., 1986), or the decrease in available hydrolysis sites because of inhibitors that bind irreversibly to the active sites of the enzyme (Zhai et al., 2018). Afterwards, the hydrolysis reaction has an ascending linear path. Guérard et al. (2001) explain this increase either by releasing peptides with a higher affinity for the enzymes, or by releasing more co-factors (a non-protein chemical compound or metal ion) that act as a catalyst and increase enzyme's activity. If the

Table 4: Pollen proteins degree of hydrolysis

Hydrolysis Time	Degree of hydrolysis %	
	Alcalase 2.4 L	Protamex
30	$5.57 \pm 0{,}806^{b}$	$6.71 \pm 0{,}806^{b}$
60	$7.88 \pm 0{,}509^{ab}$	$8.6 \pm 0{,}509^{ab}$
150	$7.53 \pm 0{,}502^{ab}$	$8.24 \pm 0{,}502^{ab}$
240	$10.39 \pm 0{,}806^{a}$	$9.31 \pm 0{,}806^{a}$

hydrolysis had lasted longer, the rate of hydrolysis would have reached the stationary phase.

It can be seen that Alcalase 2.4L demonstrated a little higher proteolytic capacity (10.39 %) than Protamex (9.31 %), although not a statistically significant difference. Alcalase 2.4L has a broad substrate specificity, having peptidase, esterase and amidase activities. Alcalase 2.4 L enzyme has a serine amino acid group at its active site, essential for substrate binding and cleavage. This catalytic center contains serine as a nucleophile, aspartate as an electrophile and histidine as a base (Novozyme A/S 2010). Between the enzyme and the substrate, a covalent bond is formed by acylation, which leads to the loss of the corresponding amino acid or peptide fragment. The nucleophilic attack of a water molecule on the covalent peptide bond between the amino and carboxyl groups of two adjacent amino acids causes the de-acylation and cleaves most of the peptide bonds within a protein molecule (Lassoued et al., 2016).

Protamex is also composed of a catalytic triad in the active site (Chang et al., 2021) and has the capacity of interacting with several types of bonds, because it could have affinity for serine, leucine or phenylalanine amino acids.

The results obtained in this study were lower than those reported in previous studies (Hu et al., 2007; Marinova et al., 2010), but similar to those obtained by Liang et al. (2020). The degree of hydrolysis is influenced by different factors: types of enzymes, enzyme specificity, pH, enzyme/substrate ratio, hydrolysis time and temperature (Liang et al., 2020). In the structure of proteins, the hydrophobic regions are partially covered by the more hydrophilic regions. Following enzymatic hydrolysis, the protein peptide bonds are cleaved, which leads to a decrease in molecular mass, to a greater accessibility to hydrophobic regions and the release of more ionizable groups. The solubility of the hydrolysates is probably due to the presence of small size molecules, and by increasing the number of exposed ionizable amino and carboxyl groups (Panyam et al., 1996). These observations may help to explain the reasons of low DH, namely: lower solubility of pollen proteins due to the insufficiently degraded outer layer of exine, which limits the access of enzymes and holds the resistance to enzymatic degradation; too short hydrolysis time, because the specificity of the enzymes to the substrate requires more time to reach the same DH of other hydrolysates. If the method used to determine DH is considered, it should be noted that TCA precipitates whole proteins and high molecular mass peptides. In this regard, the low values obtained suggest a smaller breakdown of the protein molecules with the accumulation in the supernatant of a small number of peptides with low molecular mass and amino acids (Carreira et al., 2013).

On the whole, acidic or neutral proteases (Protamex) have a lower activity than the alkaline ones of which Alcalase 2.4L is part (Klompong et al., 2007). It should be noted, however, that the activity of the enzymes was not recorded until the steady state appeared, which shows the end of the enzymatic process.

The DH directly influences the length of the peptide chain and implicitly its nutritional, functional, sensory properties and biological activity.

Bioactive peptides exhibit health effects, namely ACE – inhibitory, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antidiabetic and immunomodulatory depending on their molecular mass, amino acid composition and sequence length. ACE-inhibitory activity is related to the number of hydrophobic amino acids in the peptide sequences (especially glycine and proline) probably due to the active site of ACE being more accessible by hydrophobic peptides (Maqsoudloua et al., 2019).

The Antidiabetic activity of peptides is due to the inhibition of important enzymes namely dipeptidyl peptidase IV (DPP IV), α-amylase, and α-glucosidase. Dipeptides and tripeptides containing proline residue at the N-terminal position act by substrate type inhibition on DPP IV (peptides bind to the active site of the enzyme which is degraded into smaller fragments) (Guérard et al., 2001; Nongonierma et al., 2014). It has been observed that alpha-amylase inhibitors contain high molecular weight aromatic amino acids (phenylalanine, tryptophan, tyrosine), which also form aromatic-aromatic interactions with substrate-binding pockets of the α-amylase enzyme which has aromatic residues in addition to hydrogen bonds, electrostatic and van der Waals interactions (Siow et al., 2016). The following analysis of 43 α-glucosidase inhibitory peptides found the presence of hydroxyl or basic amino acids at the N-terminal. Also, the presence of proline in the peptide chain and alanine and methionine at the C-terminal suggests the inhibitory capacity of these peptides (Ibrahim et al., 2017).

The presence of hydrophobic amino acids (isoleucine, proline, alanine, valine) or basic acids (arginine, histidine, lysine) in the structure of peptides in the carboxyl terminal is correlated with immunomodulatory activity, proven by the increased proliferation of macrophage (Xu et al., 2019).

Determination of the MM distribution

The MM distribution shows the effect of enzymatic hydrolysis on the composition of protein subunits in pollen. The hydrolysates obtained with the two enzymes were fractionated by ultrafiltration using molecular weight cut-off membrane of 100 kDa. The two fractions obtained

were subjected to electrophoresis to establish their SDS-PAGE patterns (Fig. 1).

The logarithm of the known MM of standard and Rf for samples were plotted into a linear graph. The resulting electrophoretic profiles were analyzed and the MM of the proteins in enzymatic hydrolysates were registered. On the whole, the gel electrophoresis pattern presented few protein bands, likely because of the low protein content of enzymatic hydrolysates. Alcalase 2.4L and Protamex showed a range of low molecular and medium-size mass peptides. The differences between the MM distribution of the hydrolysates and the type of bioactive peptides produced are determined by the protease type involved, concentration, substrate specificity, protease-to-substrate ratio and incubation time (Klompong et al., 2007). The two enzymes are serine endopeptidases and act by breaking peptide bonds from non-terminal amino acids randomly into peptides with different chain lengths or amino acids (Ambigaipalan et al., 2015). BP hydrolysate obtained with Alcalase 2.4L, and which had a degree of hydrolysis of 10.39 %, showed a rapid hydrolysis and the presence of one major band with a MM of 4.8 kDa, corresponding to small peptides (lane 2). Bands above this MM disappeared possibly for two reasons: the capacity of this enzyme to hydrolyze intensely high molecular and medium MM fractions to low molecular size peptides, which are present in the sample; or the low content of proteins in the sample. BP proves to be a suitable substrate for Alcalase 2.4L whose action leads to obtaining small peptides, confirming the efficiency of proteolytic action. Fractions with MM <4 kDa were not detected by SDS-PAGE probably due to the use of an acrylamide resolving gel with a lower concentration (0.12 g mL− 1) or because MM values are a function of relative mobility, which has many variables (Asquith et al., 1993). This result is consistent with Saisavoey (Saisavoey et al., 2021) who separated fractions of 0.65, 3, 5, and 10 kDa by ultrafiltration of the BP hydrolysate obtained with Alcalase 2.4L. After testing the antioxidant activity of these peptides, he noticed that the lower their MM, the higher the antioxidant activity. Another study has demonstrated the applicability of peptides from soy protein hydrolysate obtained with Alcalase 2.4L based on their MM, namely 2-5 kDa for functional food, 1-2 kDa for sport-man or patient and below 1 kDa for allergy treatment (Nguyen et al., 2015).

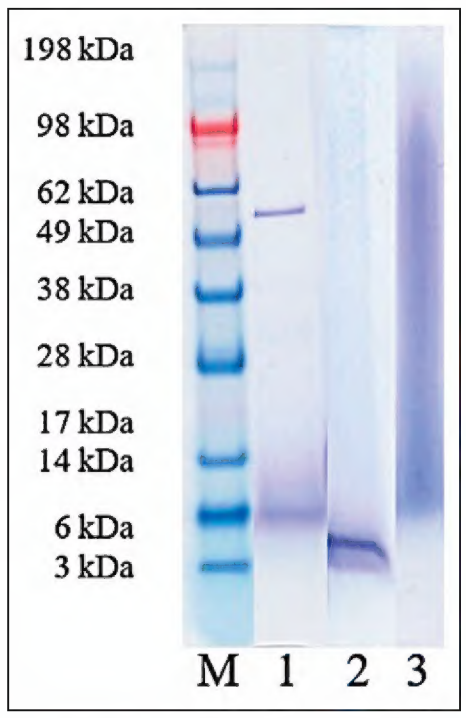

Fig 1. SDS-PAGE profiles of pollen protein hydrolysates obtained by two proteases Alcalase 2.4L and Protamex at optimized parameters. Precast gel 4-12%; 20 µL sample/well; dilution 1:1; Marker SeeBlue® Plus2 Pre-Stained Protein Standard; Lane M -molecular mass marker; lane 1- enzymatic hydrolysate obtained with Protamex, 50 0C, 1%, 4h; lane 2- enzymatic hydrolysate obtained with Alcalase 2.4L, 55 0C, 2%, 4h; lane 3 BP protein has not denatured.

Following the enzymatic hydrolysis with Protamex, which achieved a DH of 9.31 %, electrophoretic patterns of the hydrolysate displayed three distinct peptide bands in the low-to-medium MM range, corresponding to 5.8 kDa, a faint peptide band near 31 kDa and 51 kDa (lane 1). Enzymatic treatment with Protamex did not completely hydrolyze medium size MM subunits. These differences observed between the electrophoretic patterns of the two hydrolysates could result from larger proteins in the raw material that have not been partially hydrolyzed by the Protamex, which probably requires more time to cleavage the proteins into shorter chains. Recent research (Hailegiorgis et al., 2020) has sought the evaluation by electrophoresis of albumin and globulin protein subunits from the durum wheat seed varieties. In the albumin fraction there have been detected around 20 different peptides with MM ranging from 10 to 65 kDa. In the globulin fraction there were highlighted 10 to 16 different polypeptides with MM ranging from 10 to 70 kDa. The protein subunits highlighted in lane 1 fall within the ranges of albumin and globulin fractions.

In a study by Moayedi et al. (2016) it was reported that fractions from the Alcalase hydrolysate of bee pollen, corresponding to molecular weights between 1423 Da -13,000 Da have lower ACE-inhibitory activity (95%) compared to the fraction with molecular weight of 13,000 Da from a range of 3,000 to 70,000 Da, whose activity is 100%. This fact can be explained by the presence of a large amount of hydrophilic amino acids in peptides with lower molecular weights. It is known that the size of the peptides and the balance between hydrophilic-hydrophobic amino acids are important factors in ACE-inhibitory activity. The active site of ACE is more accessible for hydrophobic peptides and inaccessible to the highly hydrophilic peptides. Analysis of the ACE-inhibitory peptides profiles showed less than 20 amino acids in length. The two main hydrophobic amino acids present in the peptide sequences identified in BP hydrolysate were Glycine and Proline. Regarding antioxidant activity, higher values were recorded

for small peptides with a molecular weight below 3000 Da (Ktari et al., 2014).

In fraction 25 of the Alcalase hydrolyzed pollen, (Maqsoudloua et al., 2019) identified more than 100 peptides that possess common sequences found in ACE-inhibitory and antioxidant peptides, included in Biopep-UWM-Bioactive Peptides. The peptides SDGGGPTYGY and SPYCYG contain SDG and SPY at the N- terminal position, ACE- inhibitory tripeptides derived from bean (*Phaseolus vulgaris*). Another ACE inhibitory peptide derived from bean is AGG, a tripeptide found at the N-terminal sequence of AGGGVEDVYGEDR, and also present at the C-terminal sequence of YAGG (Mojica et al., 2015).

Regarding the enzyme-free sample (lane 3), there was a "smeared" aspect of the lane, with a darker color than the gel background, which indicated the presence of many proteins, which cannot be distinguished without them being denatured.

The SDS–PAGE analysis indicated that both enzymes are able to hydrolyze the high-MM fractions, but Alcalase 2.4L was slightly more efficient leading to small peptides.

Future development

In the last two decades, bioactive peptides from soybean, wheat germ, lupine, mung bean, rapeseed have been identified and characterized. Biological activities such as antioxidant, antihypertensive, ACE inhibitory, anti-inflammatory, immunoregulatory, antitumoral, anti-cholesterol have been reported for plant-based biopeptides prepared via enzymatic hydrolysis of proteins (Montesano et al., 2020).

The interest in other plant-derived valuable protein source containing bioactive peptides is growing. the Research of bioactive proteins in BP is still in the early stage of development. In-depth studies are needed to isolate, identify, purify BP peptides. The amino acid composition, the sequence in which the amino acids are linked and its length, posttranslational modifications, molecular masses, and mass/charge ratio (m/z) are of most importance.

Their biological functionality should be correlated with their structures and mechanism of action. Some of the amino acids positioned on the N-terminal and C-terminal of the peptides have significant properties depending on the specificity of the enzymes used.

BP peptides with a significant role in the development of novel functional ingredients/pharmaceuticals should be implemented in clinical trials to confirm the efficacy and safety of their administration. A key recommendation regarding the safe use of pollen is the individual testing of sensibility, given its potential allergenic effect.

CONCLUSION

Poly-floral BP has a complex chemical composition, rich in nutrients (proteins, lipids, macro- and micro-nutrients) and biologically active compounds (polyphenolic compounds, β-carotene), interesting from an economic point of view. BP partial hydrolysates were produced through the combination of enzymatic hydrolysis with ultrasound, that led to structural changes of proteins and the release of peptides and small peptides. The obtained results showed that Alcalase 2.4L demonstrated slightly higher proteolytic capacity (10.39 %) than Protamex (9.31 %), however there is no statistically significant difference between them. By enzymatic hydrolysis, using either of the two enzymes, the pollen proteins acquire commercial and nutritional value. The modification of the pollen structure contributes to the increase of accessibility of peptides in the body, through their more efficient absorption compared to proteins or mixtures of free amino acids. As a perspective, further investigations will be performed to separate the peptides obtained, to determine the amino acid composition and sequence, and to determine their biological activity, which may be important for health promoting effects.

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Conflict of interest

Author declares that there is no conflict of interest.

Authors' contributions

Diana Pasarin and Camelia Rovinaru: Conceptualization, Methodologies, Data analysis and interpretation, writing and correction of the manuscript

REFERENCE

Adler-Nissen, J. 1986. Enzymic Hydrolysis of Food Protein. Elsevier Applied Science Publishers, London.

Ambigaipalan, P., A. S. Al-Khalifa and F. Shahidi. 2015. Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. J. Funct. Foods. 18: 1125-1137.

Aslan, H. E. and S. Beydemir. 2017. Phenolic compounds: the inhibition effect on polyol pathway enzymes. Chem. Biol. Interact. 266: 47-55.

Aspmo, S. I., S. J. Horn and V. G. Eijsink. 2005. Enzymatic hydrolysis

of Atlantic cod (*Gadus morhua* L.) viscera. Process Biochem. 40: 1957-1966.

Asquith, T. N., R. Takigiku, M. P. Purdon, D. L. Gauggel, T. W. Keough and M. P. Lacey. 1993. Section I. Accurate determination of molecular mass for proteins isolated from polyacrylamide gels. In: Techniques in Protein Chemistry IV. Academic Press Inc, San Diego California. p. 99-106.

Block, G., R. Sinha and G. Gridley. 1994. Collection of dietary-supplement data and implication for analysis. Am. J. Clin. Nutr. 59: 232S-239S.

Bruno, S. F., T. G. Kudre and N. Bhaskar. 2019. Effects of different pretreatments and proteases on recovery, umami taste compound contents and antioxidant potentials of *Labeo rohita* head protein hydrolysates. J. Food. Sci. Tech. 56: 1966-1977.

Campos, M. G. R., S. Bogdanov, L. G. de Almeida-Muradian, T. Szczesna and Y. Mancebo. 2008. Pollen composition and standardization of analytical methods. J. Apicult. Res. 47: 154-161.

Carreira, R. L., C. B. D. Ornellas, H. A. Morais, S. Da Motta and M. P. C. Silvestre. 2013. Influence of precipitation and ultrafiltration on the peptide profile of casein hydrolysates. Science and Agrotechnology. 27: 414-421.

Chang, C., S. Gong, Z. Liu, Q. Yan and Z. Jiang. 2021. High-level expression and biochemical characterization of an alkaline serine protease from *Geobacillus stearothermophilus* to prepare antihypertensive whey protein hydrolysate. BMC Biotechnol. 21: 1-13.

Cheng, Y., Y. Liu, J. Wu, P. O. Donkor and T. Li. 2017. Improving the enzymolysis efficiency of potato protein by simultaneous dual-frequency energy-gathered ultrasound pre-treatment: Thermodynamics and kinetics. Ultrason. Sonochem. 37: 351-359.

Conte, P., A. Del Caro, F. Balestra, A. Piga and C. Fadda. 2018. Bee pollen as a functional ingredient in gluten-free bread: A physical-chemical, technological and sensory approach. LWT Food Sci. Technol. 90: 1-7.

Demir, Y. and Z. Köksal. 2019. The inhibition effects of some sulfonamides on human serum Paraoxonase-1 (hPON1). Pharmacol. Rep. 71: 545-549.

Du, M., J. Xie, B. Gong, X. Xin and T. Wei. 2017. Extraction, physicochemical characteristics and functional properties of Mung bean protein. Food Hydrocolloid. 76: 131-140.

Ezeagu, I. E., J. K. Petzke, C. C. Metges, A. O. Akinsoyinu and A. D. Ologhobo. 2002. Seed protein contents and nitrogen-to-protein conversion factors for some uncultivated tropical plant seeds. Food Chem. 78: 105-109.

Feás, X., M. P. Vázquez-Tato, L. Estevinho, J. A. Seijas and A. Iglesias. 2012. Organic bee pollen: Botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. Molecules. 17: 8359-8377.

Gaudichon, C. and J. Calvez. 2021. Determinants of amino acid bioavailability from ingested protein in relation to gut health. Curr. Opin. Clin. Nutr. Metab. Care. 24: 55-61.

Guérard, F., L. Dufosse, D. De La Broise and A. Binet. 2001. Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacores*) wastes using Alcalase. J. Mol. Catal. B. Enzym. 11: 1051-1059.

Hailegiorgis, D., F. Mekonne, F. Hailu, C. Lee and S. Yun. 2020. Composition and molecular weight distribution of albumin and globulin protein isolates from durum wheat genotypes. Am. J. Plant Sci. 11: 137-147.

Hong, G. P., S. G. Min and Y. J. Jo. 2019. Anti-oxidative and anti-aging activities of porcine by-product collagen hydrolysates produced by commercial proteases: Effect of hydrolysis and ultrafiltration. Molecules. 24: 1104.

Hu, X., Z. Xue and M. Wu. 2007. Preparation of peptides hydrolyzed from rape pollen glutelins. 12th International Rapeseed Congress: 2007 Quality, Nutrition and Processing: Processing Technology, Wuhan, China. .

Ibrahim, M. A., M. J. Bester, A. W. H. Neitz and A. R. M. Gaspar. 2017. Structural properties of bioactive peptides with α-glucosidase inhibitory activity. Chem. Biol. Drug Design. 91: 370-379.

Junior, N., R. De Almeida Barros, C. E. Vital, S. L. Barbosa and J. V. A. De Oliveira. 2020. Enzymatic Assay of Protease Using Azocasein as Substrate V.1. Available from: https://doi.org/10.17504/protocols.io.bayzifx6 on April 15, 2021

Kaewka, K., C. R. Therakulkait and K. Cadwallader. 2009. Effect of preparation conditions on composition and sensory aroma characteristics of acid hydrolyzed rice bran protein concentrate. J. Cereal Sci. 50: 56-60.

Kieliszek, M., K. Piwowarek, A. M. Kot, S. Błażejak and A. Chlebowska-Śmigiel. 2018. Pollen and bee bread as new health-oriented products: A review. Trends Food Sci. Tech. 71: 170-180.

Klompong, V., S. Benjakul, D. Kantachote and F. Shahidi. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102: 1317-1327.

Komosinska-Vassev, K., P. Olczyk, J. Kaźmierczak, L. Mencner and K. Olczyk. 2015. Bee pollen: Chemical composition and therapeutic application. Evid. Based Complement. Altern. Med. 2015: 297425.

Kostić, A. Ž., D. D. Milinèiæ, U. M. Gašiæ, N. Nediæ and S. P. Stanojeviæ. 2019. Polyphenolic profile and antioxidant properties of bee-collected pollen from sunflower (*Helianthus annuus* L.) plant. LWT Food Sci. Technol. 112: 108244.

Kostić, A. Ž., M. B. Baraæ, S. P. Stanojeviæ, D. M. Milojkoviæ-Opsenica and Ž. L. Tešiæ. 2015. Physicochemical composition and techno functional properties of bee pollen collected in Serbia. LWT Food Sci. Technol. 62: 301-309.

Kristinsson, H. G. and B. A. Rasco. 2000. Fish protein hydrolysates: Production, biochemical, and functional properties. Crit. Rev. Food Sci. 40: 43-81.

Ktari, K., R. Nasri, K. Mnafgui, K. Hamden, T. O. Boudaouara, A. Feki and M. Nasri. 2014. Antioxidative and ACE inhibitory activities of protein hydrolysates from zebra blenny (*Salaria basilisca*) in alloxan-induced diabetic rats. Process Biochem. 49: 890-897.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature. 227: 680-685.

Lassoued, I., L. Mora, A. Barkia, M. C. Aristoy and M. Nasri. 2016. Angiotensin I-converting enzyme inhibitory peptides FQPSF and LKYPI identified in *Bacillus subtilis* A26 hydrolysate of thornback ray muscle. Internet. J Food Sci. Technol. 51: 1604-1609.

Li, Q. Q., K. Wang, M. C. Marcucci, A. C. H. Sawaya and L. Hu. 2018. Nutrient-rich bee pollen: A treasure trove of active natural metabolites. J. Funct. Foods. 49: 472-484.

Liang, X., H. Yang, J. Sun, J. Cheng and X. Luo. 2020. Effects of enzymatic treatments on the hydrolysis and antigenicity reduction of natural cow milk. Food Sci. Nutr. 9: 985-993.

Liolios, V., C. Tananaki, A. Papaioannou, D. Kanelis and M. A. Rodopoulou. 2019. Mineral content in mono-floral bee pollen: Investigation of the effect of the botanical and geographical origin. J. Food Measure. Character. 13: 1674-1682.

Maqsoudloua, A., A. S. Mahoonaka, L. Morab, H. Mohebodinic and F. Toldráb. 2019. Peptide identification in Alcalase Hydrolysate pollen and comparison of its bioactivity with royal jelly. Food Res. Int. 116: 905-915.

Margaoan, R., L. A. Marghitas, D. S. Dezmirean, O. Bobis and C. M. Mihai. 2012. Physical-chemical composition of fresh bee pollen from Transylvania. Bull UASVM. Anim. Sci. Biotechnol. 69: 1-5.

Marghitas, L. A., C. Pasca, D. S. Dezmirean, O. Bobiș and V. Bonta. 2016. Antibacterial activity of pollen extracts on pathogenic microflora from milk. Bull UASVM. Anim. Sci. Biotechnol. 73: 261-262.

Marinova, M. D., P. Bozhidar and P. Tchorbanov. 2010. Preparation of antioxidant enzymatic hydrolysates from honeybee-collected pollen using plant enzymes. Enzyme Res. 2010: 415949.

Millward, D. J. 2012. Amino acid scoring patterns for protein quality assessment. Br. J. Nutr. 108: 31-43.

Moayedi, A., L. Mora, C. M. Aristoy, M. Hashemi, M. Safari and M. Toldrá. 2016. ACE inhibitory and antioxidant activities of peptide fragments obtained from tomato processing by-products fermented using bacillus subtilis: Effect of amino acid composition and peptides molecular mass distribution. Appl. Biochem. Biotechnol. 181: 48-64.

Mojica, L., K. Chen and E. G. Mejia. 2015. Impact of commercial precooking of common bean (*Phaseolus vlgaris*) on the generation of peptides, after pepsin-pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase-IV. J Food Sci. 80: 188-198.

Montesano, D., M. Gallo, F. Blasi, L. Cossignani. 2020. Biopeptides from vegetable proteins: New scientific evidences. Curr. Opin. Food Sci. 31: 31-37.

Nchienzia, H. A., R. O. Morawicki and V. P. Gadang. 2010. Enzymatic hydrolysis of poultry meal with endo-and exopeptidases. Poult. Sci. 89: 2273-2280.

Nguyen, H. T. M., K. S. B. Sylla, Z. Randriamahatody, C. Donnay-Moreno, J. Moreau and L. T. Tran. 2011. Enzymatic hydrolysis of yellowfin tuna (*Thunnus albacares*) by-products using protamex protease. Food Technol. Biotechnol. 49: 48-55.

Nguyen, P. M. 2015. Alcalase and protamex hydrolysis of bioactive peptides from soybean. Bull. Enviroin. Pharmacol. Life Sci. 4: 132-143.

Nongonierma, A. B. and R. J. FitzGerald. 2014. Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis. Food Chem. 145: 845-852.

Panyam, D. and A. Kilara. 1996. Enhancing the functionality of food proteins by enzymatic modification. Trends Food Sci. Technol. 7: 120-125.

Rutherfurd, S. M. 2010. Methodology for determining degree of hydrolysis of proteins in hydrolysates: A review. J. AOAC. Int. 93: 1515-1522.

Saisavoey, T., P. Sangtanoo, P. Srimongkol, O. Reamtong and A. Karnchanatat. 2021. Hydrolysates from bee pollen could induce apoptosis in human bronchogenic carcinoma cells (ChaGo-K-1). J. Food Sci. Technol. 58: 752-763.

Siow, H. L. and C. Y. Gan. 2016. Extraction, identification, and structure-activity relationship of antioxidative and α-amylase inhibitory peptides from cumin seeds (*Cuminum cyminum*). J. Funct. Foods. 22: 1-12.

Soussi, N., A. Bougatef, Y. Triki-Ellouz and M. Nasri. 2007. Biochemical and functional properties of Sardinella (*Sardinella aurita*) by-product hydrolysates. Food Technol. Biotechnol. 45: 187-194.

Spulber, R., M. G. Vladu, O. Popa and N. Babeanu. 2017. Phenolic content and potential inhibitory activity of Romanian bee pollen on different plant pathogenic strains. Sci. Bull. Ser. F. Biotechnol. 21: 104-108.

Stahl, W. and H. Sies. 2003. Antioxidant activity of carotenoids. Mol. Aspects Med. 24: 345-351.

Szucs, M., M. Angulo, C. Costa and M. Márquez. 2021. Meat waste valorization through protein hydrolysis using different types of proteases. Recent Prog. Mater. 3: 045.

Taha, E. K. A., S. Al. Kahtani and R. Taha. 2019. Protein content and amino acids composition of bee-pollens from major floral sources in Al-Ahsa, Eastern Saudi Arabia. Saudi J. Biol. Sci. 26: 232-237.

Thakur, M. and V. Nanda. 2018. Assessment of physical-chemical properties, fatty acid, amino acid and mineral profile of bee pollen from India with a multivariate perspective. J. Food Nutr. Res. 57: 328-340.

Thakur, M. and V. Nanda. 2020. Composition and functionality of bee pollen: A review. Trends Food Sci. Tech. 98: 82-106.

Venkatachalam, K. and M. Nagarajan. 2019. Assessment of different proteases on degree of hydrolysis, functional properties and radical scavenging activities of salted duck egg white hydrolysate. J. Food Sci. Technol. 56: 3137-3144.

Xu, Z., T. M. Mao, L. Huang, Z. C. Yu and B. Yin. 2019. Purification and identification immunomodulatory peptide from rice protein hydrolysates. Food Agric. Immunol. 30: 150-162.

Zhai, R., J. Hu and J. N. Saddler. 2018. Understanding the slowdown of whole slurry hydrolysis of steam pre-treated lignocellulosic woody biomass catalyzed by an up-to-date enzyme cocktail. Sustain. Energy Fuels. 2: 1048-1056.

Zhang, Z., S. He, H. Liu, X. Sun and H. Sun. 2020. Effect of pH regulation on the components and functional properties of proteins isolated from cold-pressed rapeseed meal through alkaline extraction and acid precipitation. Food Chem. 327: 126998.

Zheng, X. Q., J. T. Wang, X. L. Liu, Y. Sun and Y. J. Zheng. 2015. Effect of hydrolysis time on the physicochemical and functional properties of corn glutelin by Protamex hydrolysis. Food Chem. 172: 407-415.

Zuluaga, C., A. Martínez, J. Fernández, J. López-Baldó and A. Quiles. 2016. Effect of high-pressure processing on carotenoid and phenolic compounds, antioxidant capacity, and microbial counts of bee-pollen paste and bee-pollen-based beverage. Innov. Food Sci. Emerg. Technol. 37: 10-17.

Zuluaga-Domínguez, C., L. Castro-Mercado and M. C. Quicazán. 2019. Effect of enzymatic hydrolysis on structural characteristics and bioactive composition of bee-pollen. J. Food Process. Preserv. 43: 1-11.